The Transcription Factor FoxK Participates with Nup98 To Regulate Antiviral Gene Expression

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ABSTRACT  Upon infection, pathogen recognition leads to a rapidly activated gene expression program that induces antimicrobial effectors to clear the invader. We recently found that Nup98 regulates the expression of a subset of rapidly activated antiviral genes to restrict disparate RNA virus infections in Drosophila by promoting RNA polymerase occupancy at the promoters of these antiviral genes. How Nup98 specifically targets these loci was unclear; however, it is known that Nup98 participates with transcription factors to regulate developmental-gene activation. We reasoned that additional transcription factors may facilitate the Nup98-dependent expression of antiviral genes. In a genome-wide RNA interference (RNAi) screen, we identified a relatively understudied forkhead transcription factor, FoxK, as active against Sindbis virus (SINV) in Drosophila. Here we find that FoxK is active against the panel of viruses that are restricted by Nup98, including SINV and vesicular stomatitis virus (VSV). Mechanistically, we show that FoxK coordinately regulates the Nup98-dependent expression of antiviral genes. Depletion of FoxK significantly reduces Nup98-dependent induction of antiviral genes and reduces the expression of a forkhead response element-containing luciferase reporter. Together, these data show that FoxK-mediated activation of gene expression is Nup98 dependent. We extended our studies to mammalian cells and found that the mammalian ortholog FOXK1 is antiviral against two disparate RNA viruses, SINV and VSV, in human cells. Interestingly, FOXK1 also plays a role in the expression of antiviral genes in mammals: depletion of FOXK1 attenuates virus-inducible interferon-stimulated response element (ISRE) reporter expression. Overall, our results demonstrate a novel role for FOXK1 in regulating the expression of antiviral genes, from insects to humans.

IMPORTANCE  Innate immunity is characterized by rapid gene expression programs, from insects to mammals. Furthermore, we find that Nup98, known for its roles in the nuclear pore, plays a noncanonical role in binding the promoters and poising a subset of loci for rapid antiviral gene induction. It was unclear how Nup98 accesses these specific genes, and we here demonstrate that Nup98 cooperates with the transcription factor FoxK to regulate this gene expression program. Depletion of FoxK specifically reduces the induction of Nup98-dependent genes. Further, we find that the antiviral function of FoxK is conserved, as the human ortholog FOXK1 is also antiviral and regulates gene expression from virus-induced promoters. Although other forkhead transcription factors have been implicated in immunity, a role for FoxK in antiviral defense was previously unappreciated. Our findings reveal a conserved and novel role for FoxK in coordinating with Nup98 to promote a robust and complex antiviral transcriptional response.

Innate immunity plays an evolutionarily conserved role in the defense against invading pathogens. Upon sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), signaling pathways are engaged and activate antimicrobial gene expression programs. In mammals, virus recognition induces type I interferons (IFNs), which then activate hundreds of downstream interferon-stimulated genes (ISGs) from interferon-stimulated response elements (ISREs) encoding antiviral effector proteins (1–3). These gene expression programs are tightly orchestrated to regulate specific downstream immune responses (4, 5). Like mammals, insects induce a rapid antimicrobial gene expression program resulting in the induction of large numbers of antiviral effectors (6–8).

In both cases, there is sequential activation; primary response genes are induced first and are translation independent, while secondary response genes depend on new translation. Furthermore, some primary response genes are regulated at the level of transcriptional initiation, while some of the most rapidly induced genes are regulated downstream of initiation at the step of transcriptional elongation by transcriptional pausing (9–12). In Drosophila, the rapidly activated antimicrobial gene expression program has this complexity with primary and secondary response genes (8, 13, 14). Furthermore, we demonstrated that transcriptional pausing regulates half of the antiviral gene expression program and that a subset is dependent on Nup98 (7, 8).

Mechanistically, we found that Nup98 binds to the promoters...
of these rapidly inducible antiviral genes and positively regulates the activity of RNA polymerase II (RNAP II) at these loci (7). Nup98 is known to coordinate with transcription factors such as GAGA factor, YY1, and MBD-R2 to access specific promoters for developmental-gene induction (15, 16). However, whether Nup98 participates with such transcription factors to regulate antiviral gene expression is not known. Therefore, we set out to identify transcription factors that may play a role in regulating antiviral gene expression. In a genome-wide RNA interference (RNAi) screen, we validated 37 antiviral genes, including 5 genes associated with transcription and one transcription factor, FoxK, active against Sindbis virus (SINV) (17). Forkhead proteins are characterized by a conserved DNA binding domain known as the forkhead domain (18). There are 18 forkhead genes in Drosophila and 50 in humans (19). FoxK belongs to the K subfamily of Fox genes and is characterized by the presence of both a forkhead DNA binding domain and an additional forkhead-associated (FHA) domain (20). Fox transcription factors are evolutionarily conserved, with roles in development, aging, cell cycle, cancer, and immunity (19, 21–24). One forkhead gene, the Foxo gene, plays important roles in antimicrobial gene expression in Drosophila (24). In Drosophila, FoxK is essential for embryonic midgut development and is required for transforming growth factor beta (TGF-β) signaling in the embryonic midgut endoderm (25). Mammals have two orthologs, FOXK1 and FOXK2. The best-studied role for FoxK is as a transcriptional repressor in myogenic cells, where FoxK1 countersacts the activities of other transcription factors, such as Foxo4, Me2, or Foxo3, represses SRF-dependent genes, and interacts with the Sin3-Sds3 repressor complex (26–29). FOXK1 and FOXK2 also regulate starvation-induced atrophy and autophagy programs (30). A proteomic study showed that FOXK1 and FOXK2 interact with IRF2 and IRF4 and modestly potentiate expression from the beta interferon (IFN-β) promoter (31), but they have not been explored more extensively. Here we demonstrate a role for FoxK in restricting virus infection in Drosophila: FoxK is active against disparate RNA viruses, including human arboviruses. Mechanistically, we found that FoxK regulates the expression of a subset of rapidly induced antiviral genes. Indeed, FoxK is required for virus-induced gene expression of Nup98-dependent genes but not Nup98-independent genes. In a direct transactivation assay, we found that FoxK-dependent stimulation of a forkhead response element-containing (FHRE) reporter depends on Nup98. Overall, these results suggest that FoxK participates with Nup98 to directly regulate antiviral gene expression. Because FoxK is a highly conserved gene, we extended our studies to human cells. In human cells, we found that FOXK1 is active against SINV and vesicular stomatitis virus (VSV), which are also controlled by FoxK in Drosophila. Furthermore, we tested whether FOXK1 impaired virus-induced gene expression and found that depletion of FOXK1 led to significantly reduced expression of an ISRE-luciferase (ISRE-Luc) reporter upon viral challenge. Taken together, these results suggest that FoxK plays a conserved role in regulating the expression of antiviral genes. These findings shed light on a previously unidentified requirement for FoxK in antiviral defense via its transcriptional regulation of antiviral genes and its coordination with Nup98 to restrict viral infection.

RESULTS

FoxK is active against SINV in vitro and in adult flies. We recently completed a genome-wide RNAi screen and validated 37 antiviral genes active against the human alphavirus SINV, which is a single-stranded positive-sense virus (17). Among these genes was the forkhead transcription factor FoxK. Since forkhead transcription factors have been implicated in innate immune gene expression, we set out to study the role of FoxK in antiviral defense (19, 21, 23). Using an independent double-stranded RNA (dsRNA), we further confirmed our screen results and found that depletion of FoxK significantly enhanced SINV infection as measured by automated microscopy and quantification (Fig. 1A and B). We also performed immunoblot analysis and found that endogenous FoxK was efficiently depleted but that SINV gene expression was significantly increased (Fig. 1C and D). We further validated our results using several nonoverlapping RNAi reagents and found that FoxK was reduced by multiple independent dsRNAs, and this resulted in enhanced SINV replication (Fig. 1E). These results establish an antiviral role for FoxK in Drosophila cells.

To determine whether FoxK plays an antiviral role at the organismal level, we generated flies deficient for FoxK using in vivo RNAi driven by a ubiquitous but low-level promoter (daughterless [da] GAL4). We challenged the control flies as well as FoxK-depleted flies with SINV and monitored virus replication by immunoblotting. We observed that FoxK-depleted flies have increased levels of viral replication (Fig. 1F). All together, these results suggest an antiviral role for FoxK against SINV in cell culture and in adult flies.

FoxK is active against disparate RNA viruses. Next we examined the role for FoxK in viral infection against additional RNA viruses that are diverse and that we have previously studied in Drosophila (7, 17, 32). First, we infected FoxK-depleted cells with the nonsegmented negative-sense rhabdovirus VSV that expresses green fluorescent protein (GFP) and observed an increase in viral gene expression as detected by immunoblotting (Fig. 2A). Second, we tested the bunyavirus Rift Valley fever virus (RVFV), which is a segmented negative-strand RNA virus. RVFV causes significant morbidity and mortality in livestock and humans (33). As with SINV and VSV, we observed increased levels of RVFV gene expression as detected by immunoblotting (Fig. 2B). Since these viruses do not naturally infect Drosophila, we examined the role for FoxK in the infection of a picorna-like virus that is a natural Drosophila pathogen, Drosophila C virus (DCV). We found that cells depleted of FoxK were also more susceptible to DCV infection than wild-type cells (Fig. 2C). Taken together, these results suggest that FoxK regulates virus infection against a broad range of RNA viruses.

FoxK regulates the expression of virally induced antiviral genes. Since FoxK is a transcription factor, we reasoned that it may play a role in antiviral gene expression. We began by assessing the SINV-induced gene expression program that we previously characterized and tested whether depletion of FoxK impacted gene expression of either Nup98-dependent (7) or Nup98-independent (see Fig. S1 in the supplemental material) genes. We tested the induction of seven Nup98-dependent genes (Fig. 3A to G) and three Nup98-independent genes (Fig. 3H to J) upon SINV infection at 2 h postinfection and found that FoxK was required for the virus-induced expression of only the Nup98-dependent genes (Fig. 3A to J). These results suggest that FoxK participates with Nup98 to regulate the expression of antiviral genes.

FoxK and Nup98 cooperate to regulate gene expression. Forkhead proteins, including FoxK, directly bind to forkhead re-
Response elements (FHRE) to regulate gene expression (34). We used a previously characterized FHRE-luciferase (FHRE-Luc) reporter plasmid that contains 6 copies of the consensus forkhead binding site and ectopically expressed FoxK (25, 35). Expression of FoxK enhanced luciferase activity from the FHRE reporter (Fig. 4A), consistent with the results of previous studies (25). Next, we examined whether Nup98 was required for this transactivation. First, we verified that depletion of Nup98 did not impact the endogenous levels of FoxK protein (Fig. S2). Next, we tested the role of Nup98 in FoxK-dependent gene expression. Indeed, when we knocked down Nup98, we did not observe the FoxK-dependent increase in luciferase activity (Fig. 4A). Altogether, these results suggest that Nup98 is required for FoxK-dependent gene induction.

Since virus infection leads to increased FoxK-dependent gene expression (Fig. 3A), we tested whether infection induces FoxK activity by monitoring expression from the FHRE promoter. We found that upon SINV infection, the FHRE reporter was induced to levels similar to the levels induced by ectopic FoxK expression (Fig. 4B). Furthermore, transactivation of the FHRE reporter was significantly enhanced in SINV-infected, FoxK-expressing cells compared to that in mock-infected cells. Overall, these results suggest that SINV infection enhances FoxK-dependent gene expression and that FoxK participates with Nup98 to regulate antiviral gene expression.

**FOXK1 regulates virus infection in human cells.** While forkhead proteins regulate gene expression in diverse physiological contexts, only a few are known to participate in innate immune gene expression programs (22, 24). Depletion of FOXK1 and FOXK2 together led to a modest potentiation of virus-induced IFN-β reporter production (~2-fold) in human HEK-293 cells using pooled small interfering RNAs (siRNAs) (31). Therefore, we explored a role for FOXK1 in viral infection. Using siRNAs, we depleted human U2OS cells of either FOXK1 or FOXK2, infected these cells with SINV, and monitored infection by microscopy. We found that depletion of SINV is significantly enhanced in cells depleted of FOXK1 (Fig. 5A and B). FOXK2 depletion showed a more modest phenotype (data not shown) and thus was not pursued. We also observed increased SINV gene expression by immunoblotting as well as increased virus production as measured by plaque assay upon FOXK1 depletion in U2OS cells (Fig. 5C and D). To further confirm the requirement for FOXK1, we used three independent siRNAs and validated robust knockdown of FOXK1 and increased SINV infection (Fig. 5E). Since we found that FoxK was active against disparate viruses, including VSV in *Drosophila*,...
we explored the role of FOXK1 in VSV infection in human cells. Indeed, we found that FOXK1 depletion led to increased VSV infection in U2OS cells by microscopy (Fig. 5F and G). We also tested another human cell line, HEK-293T cells, and found that SINV and VSV gene expression was enhanced when we depleted FOXK1 in these cells (Fig. 5H and I). Taken together, these results suggest that from flies to humans, FoxK proteins are antiviral.

**FOXK1 accumulates in the nucleus upon infection.** Since many antiviral factors are induced upon infection, we determined if the level of FOXK1 is altered upon infection. We observed an
increase in FOXK1 protein levels 2 h after SINV infection (Fig. 6A). Forkhead proteins are regulated; the active transcription factor is nuclear. Therefore, we examined the subcellular localization of FOXK1 upon infection by nucleo-cytoplasmic fractionation. While cytoplasmic FOXK1 was unchanged by infection, nuclear FOXK1 levels increased upon SINV infection (Fig. 6A). These results show that FOXK1 is induced and accumulates in the nucleus upon SINV infection in human cells.

FOXK1 regulates antiviral gene expression. We observed that FOXK1 accumulates in the nucleus upon SINV infection and that FOXK1 is antiviral. These data suggest that FOXK1 may also regulate antiviral gene expression in human cells. Upon initial viral infection, sensing by RIG-I-like receptors activates mitochondrial antiviral signaling protein (MAVS)-dependent induction of the IFN-β promoter. This leads to the production of IFN-β, which then induces hundreds of antiviral ISGs that are controlled by ISREs. Therefore, we tested the induction of IFN-β–luciferase or ISRE-luciferase reporters in the presence or absence of FOXK1. For these studies, we stimulated cells with either poly(I·C) or Sendai virus (SeV), as these are potent inducers of this process (36). We observed that in both poly(I·C)- and Sendai virus-stimulated cells, FOXK1 depletion resulted in no statistically significant change in IFN-β reporter levels (Fig. 6B and C). In contrast and importantly, knockdown of FOXK1 led to significantly reduced expression of the ISRE-Luc reporter (Fig. 6D and E). Taken together, these results suggest that FOXK1 is required for optimal expression of ISGs and that this reduced level of ISRE activity may explain the antiviral phenotype that we observed.

DISCUSSION

The Fox family of transcription factors is conserved from Drosophila to mammals, and these transcription factors have central roles in development, aging, cell cycle, cancer, and immunity (19, 21–24). Although some of the Fox genes have been well studied, little is known about the functions of the FoxK group in immunity. We present here a previously undescribed role for FoxK in restricting virus infection in Drosophila. We demonstrated that FoxK regulates the expression of a subset of virally inducible antiviral genes and cooperates with Nup98 in regulating the expression of these antiviral genes. Furthermore, we found that in human cells, FOXK1 also plays a role in antiviral defense. FOXK1 is required for optimal expression of IFN-stimulated genes by positively regulating the expression of ISREs.

Virus infection leads to the rapid activation of gene expression (7, 37, 38); however, our current understanding of this regulation is incomplete. We previously found that Nup98 is required for the positive regulation of a subset of virus-induced antiviral genes (7). Mechanistically, we demonstrated that Nup98 binds to the promoters of these genes and promotes RNAP II S5P occupancy, poised them for activation upon virus infection (7). However, the molecular mechanism by which Nup98 regulates gene expression at these loci remained unknown. Nup98 has been found to activate gene expression in other contexts, such as development (39, 40). Based on chromatin immunoprecipitation sequencing (ChIP Seq) analyses, human NUP98 was shown to associate with conserved motifs, suggesting that NUP98 participates with a number of transcription factors, such as SP1, NURD, YY1, and GAGA, to regulate developmental-gene expression (16). In Drosophila, Nup98 was found to associate with nucleosome remodeling factor (NURF), which recruits Nup98 to the promoter of a subset of developmentally regulated genes (15). We found that Nup98 cooperates with the transcription factor FoxK to regulate the expression of virus-induced genes. Moreover, using a more direct trans-
activation assay, we found that FoxK-mediated gene activation on a minimal promoter requires Nup98. Further, we found that virus infection itself activates FoxK activity on the minimal promoter. FoxK has been shown to bind to chromatin (25, 26, 28), and using the FoxK consensus sequence [(G/A)(T/C)(A/C)AA(C/T)A], we analyzed the promoters of CG9008, CG7458, and mol, which are Nup98- and FoxK-dependent virus-induced genes, and found multiple consensus FoxK binding sites (data not shown). These data suggest a model where FoxK binding to these promoters recruits Nup98, which regulates the occupancy of the active form of RNAP II (serine 5 phosphorylated) at these target genes. Indeed, it has been shown that in the yeast mutant gene for Fkh1p (ortholog of FoxK), there is a reduction in the accumulation of the Ser5 phosphorylated form of RNAP II at the promoter of Fkh1p-regulated genes (41). This demonstrates a link between early transcription events and Fkh1p. It is also possible that additional factors participate with Nup98 to regulate antiviral genes. Therefore, we examined other antiviral factors from the screen with roles in gene regulation for a requirement in Nup98-dependent gene expression (17). We depleted med30, the TF2B gene, e(y)1, and sin and found that none of these genes were required for virus-induced expression of CG9008 (Fig. S3). This shows clear specificity for FoxK in regulating these virus-stimulated genes.

Next, we extended our studies to explore the role of human FOXK1 in antiviral defense and gene expression. Indeed, we found that depletion of FOXK1 led to increased replication of SINV and VSV in two disparate human cell lines. Furthermore, we found that FOXK1 nuclear localization increases upon infection, suggesting the stimulation of this factor. Next we tested whether FOXK1 impacted the expression of two canonical downstream targets: the IFN-β promoter and ISREs. We found that depletion of FOXK1 had a statistically insignificant effect on the IFN-β reporter. A recent publication presented evidence that FOXK1 overexpression reduced and depletion of FOXK1 modestly (~2-fold) enhanced IFN-β promoter activity (31). Since we did not observe a significant change and since it was the opposite of what we predict would be the role of an antiviral factor, we next examined expression from an ISRE reporter and found that depletion of FOXK1 significantly attenuates ISRE reporter expression. This is consistent with our observed antiviral phenotype. To further explore a role for FOXK1 in antiviral–gene expression, we analyzed a recent study that observed that Foxk1 binds ~7,000 genes, includ-
ing atrophy- and autophagy-related genes, and that depletion of Foxk1 leads to decreased expression of these genes (30). We analyzed the top 1,500 candidates and found that the levels of immunity-related genes, such as the Ddx58 (RIG-I), Ifit2 (ISG54), Ifnar1, and Ifitm1 (IFI17) genes, are significantly reduced upon Foxk1 depletion. Furthermore, we determined that approximately one-third of the Nup98-bound genes derived from CHIP Seq analysis in Drosophila cells that have mouse orthologs were FoxK1 bound and regulated (30, 39), which is highly significant ($P = 1.85754\times 10^{-69}$) (see Fig. S4 in the supplemental material).

Therefore, there is striking overlap between Nup98 and FoxK1 target genes. Moreover, these studies revealed that additional transcription factor binding sites are enriched within 100 bp of Foxk1 binding sites, including AP1 sites, suggesting that Foxk1 may cooperate with other transcription factors in the regulation of specific gene expression networks (30). The presence of FOXK1 binding sites at the promoters of antiviral genes that overlap Nup98 adds a layer of complexity to the complex gene expression program regulating viral infection; future studies will reveal the mechanism by which FOXK1 regulates the antiviral transcriptional response.

In addition to finding roles for FoxK and Nup98 in the basal activation state of a subset of virus-induced genes, we found that the levels of both FoxK and Nup98 proteins are increased by infection (7). Furthermore, we observed increased nuclear localization of FoxK upon infection. Since FOXK1 and other forkhead transcription factors are regulated by phosphorylation, it is likely that virus infection induces dephosphorylation to activate nuclear translocation (30, 42–44). Nup98 is also regulated by phosphorylation (45); however, it is unknown how various phospho forms regulate gene expression. These translation-dependent changes and signal-dependent changes in FoxK and Nup98 likely regulate downstream secondary responses to viral infection. This may impact transcriptional memory, as NUP98 has been shown to control IFN-γ-dependent memory in HeLa cells (46). In conclusion, identification of FoxK introduces a new player to the existing group of molecules that regulate antiviral gene expression and highlights the elaborate gene networks involved in the regulation in antiviral responses.

MATERIALS AND METHODS:

Cells, viruses, and reagents. DL1 cells were maintained in Schneider’s medium (Invitrogen-Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. U2OS and 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen-Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. SINV-GFP was generated in BHK-21 cells and propagated in C6/36 cells (47). VSV-GFP was propagated in BHK-21 cells (48). The MP-12 strain of RVFV was propagated in BHK-21 cells (49), and DCV was propagated in DL2 cells (50). Virus titers were
determined in BHK-21 cells. Drosophila FoxK antibody and FoxK expression plasmids were reported previously (25). Anti-DCV antibody has been reported previously (50). Anti-RVFV glycoprotein Gn monoclonal antibody (MAb) 4D4 mapping to amino acids 229 to 239 of mature Gn has been described previously (51) and was a kind gift from C. Schmaljohn, USAMRIID. IFN-β and ISRE-luciferase reporters have been reported previously (52). Sendai virus (Cantell strain) was obtained from Charles River Laboratory. Poly(I:C) was purchased from Sigma. The following antibodies were also purchased: anti-GFP (SC-9996; Santa Cruz), anti-actin (SC-47778; Santa Cruz), anti-tubulin (T6199, Sigma), anti-lamin (ab-16048; Abcam), anti-FOXK1 (12025; Cell Signaling), horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham), and Alexa Fluor-conjugated secondary antibodies (Life Technologies). Firefly luciferase reagent britelite plus (catalog no. 6066761) was obtained from PerkinElmer.

**RNAi and virus infection in Drosophila cells.** The following dsRNAs were used. For FoxK, dsRNAs were custom synthesized corresponding to the catalog numbers DRSC 24971, DRSC 37606, and DRSC 37984 of the Drosophila RNAi Screening Center (DRSC). Further detail about the dsRNAs can be obtained from the DRSC website (http://www.flyrnai.org/cgi-bin/RNAi_gene_lookup_public.pl). RNAi was performed as described previously (17). Briefly, DL1 cells (18,000/well) were seeded into 384-well plates prearrayed with 250 ng/well dsRNA in 10 μl of serum-free medium. One hour later, 20 μl of complete medium was added. For RNAi in the 12-well format, DL1 cells (1 x 10^6) in 500 μl of serum-free medium was incubated with 2 μg of dsRNA. After 1 h, 1 ml of complete medium was added and cells were incubated for 72 h more to achieve gene knockdown. Drosophila DL1 cells were infected with SINV at a multiplicity of infection (MOI) of 5 for 40 h, VSV at an MOI of 0.5 for 40 h, RVFV (MP12) at MOI of 0.1 for 40 h, and DCV such that 10 to 20% of the cells were infected at 24 h postinfection.

**RNAi and virus infection in human cells.** The following siRNAs were used: catalog number s48135 from Ambion and SASI_Hs01_00149056 and SASI_Hs01_00149058 from Sigma. U2OS or 293T cells in the 6-well format were transfected with a pool of two independent siRNAs for FOXK1 (s48135 [Ambion] and SASI_Hs01_00149056 [Sigma]) or with singles (Fig. 5E) using HiPerFect (Qiagen, CA) at 20 nM according to the manufacturer’s instructions. At 72 h posttransfection, cells were replated in a 96-well format (30,000 cells per well) for infection or replated in a 6-well plate (300,000 cells per well) for virus infection. U2OS cells were infected with SINV at an MOI of 0.2 or with VSV at an MOI of 0.1 and processed for automated microscopy or immunoblotting at 14 h postinfection. HEK-293T cells were infected with SINV and VSV at an MOI of 0.02 for 14 h. For automated microscopy and image analysis, 4 wells per condition and 4 to 6 sites/well from three independent experiments were analyzed.

**Immunoblotting.** Cells were harvested in radioimmunoprecipitation assay (RIPA) buffer and processed for immunoblotting. Total protein was determined by Bradford protein assay, and equal amounts were separated on an SDS-PAGE gel. The results of representative experiments from at least three replicates are shown.

**Adult fly infection.** FoxK RNAi transgenics (stock no. 27994) were obtained from the Bloomington Stock Center. These were crossed to daGal4 (gift from the Perrimon lab), and the adult progeny (4 to 7 days old) were challenged as described previously (50).

**Luciferase assay.** DL1 cells were transfected with the 6FH-Luc reporter plasmid (obtained from Addgene [catalog number 7989]) with or without FoxK expression plasmid for 48 h. Cells were counted, and 100,000 cells were suspended in 100 μl phosphate-buffered saline (PBS) and plated per well in white, clear-bottom plates (Corning). One hundred microliters of britelite firefly luciferase reagent was added to each well, incubated for 2 to 3 min, and assayed on a luminometer (Molecular Devices).

**Cellular fractionation.** Cellular fractionation was performed as described previously (7). Briefly, U2OS cells (3 x 10^6) were resuspended in cytoplasmic lysis buffer (30 mM HEPES, 2 mM magnesium acetate, 0.1% NP-40, 5 mM dithiothreitol [DTT], protease inhibitors, and phenylmethylsulfonyl fluoride [PMSF]), lysed by passing them through a 30-gauge needle, and centrifuged at 1,000 x g for 5 min to obtain the cytoplasmic supernatant. The nuclear pellet was washed twice with wash buffer (30 mM HEPES, 2 mM magnesium acetate, 0.1% NP-40, 5 mM DTT, and PMSF), subsequently lysed in RIPA buffer, and centrifuged at 15,000 x g for 15 min, and the nuclear supernatant was collected.

**RT-qPCR.** RNA was extracted using Trizol and reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) (28025-013; Life Technologies). For quantitative PCR (qPCR), cDNA was subjected to PCR using SYBR green (4367659; Life Technologies) analyzed by the ΔΔCt method, where Ct is threshold cycle, and normalized to Rp49. Data are represented as levels of mRNA expression relative to that of the control samples and are displayed as the means ± standard deviations (SD) of results from at least three independent experiments.

**Primers used.** Primers used for RT-qPCR have been reported previously (7). In addition, the following primers were used: gnf1 (5’ CAAAG CTAAATTGGGAGGAGAAG 3’), grn (5’ CGTTTGCCGACTCTGCTGATAAC 3’), PGRP-LB F (5’ GCTGATCGGAGATTGGAACAC 3’), PGRP-LB R (5’ CCTTAGGCGGCAATGTAAGT 3’), MSI F (5’ GCATGGGCGATGTGCTTGA 3’), and MSI R (5’ GCTAGTGTTGTGTGGGTGTAG 3’).

**Statistical analysis.** Student’s t test was performed when two conditions were compared, and a one-way analysis of variance (ANOVA) was applied when multiple comparisons were made. Experiments were performed at least three times. A P value of <0.05 in each independent experiment was considered significant. The chi-square test was performed to determine the significance of overlap in the ChIP data sets.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl doi:10.1128/mBio.02509-14/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.1 MB.

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